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PLA-HA scaffolds: preparation and bioactivity

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Abstract

The photocurable polylactide (PLA) and hydroxyapatite (HA) composites were prepared for solid free-form fabrication of three-dimensional scaffolds by stereolithography (SLA). The influence of varying pore feature and HA amount of PLA/HA scaffolds on cell growth and differentiation was evaluated with MC3T3-E1 pre-osteoblasts. Morphology of adherent cells on scaffolds was examined by SEM. Alamar Blue assay was employed to observe proliferation and cytotoxicity of cells on scaffold. Alkaline phosphatase activity (ALP) and calcium content in cell-cultured samples were also measured to assess osteoblast activity. The cellular responses to various types of scaffolds at a 21-day culture period were found similar while the ALP activity observed at a 7-day culture period appeared to be superior in the scaffold with smaller pore size and higher amount of HA. These results indicated that HA and pore size had an effect on bioactivity of scaffold only at the early state of osteoblastic differentiation. At the longer culture period, all types of scaffolds showed equally cell proliferation and function, and thus demonstrated their potential to be used in bone repair application.

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Keywords: polylactide; hydroxyapatite; stereolithography; scaffold; bioactivity.

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1. Introduction

The increasing trend of research in the field of bone regeneration is driven by the need for repairing bone defects resulted from infections, tumors or bone loss by trauma. Although various kinds of materials including metals, polymers and bioceramics have been evaluated for their potential to be bone substitute, their performances are still far from those of natural bone. In recent years, polymer/bioceramic composites particularly degradable polylactide and hydroxyapatite (PLA-HA) composites have been an attractive candidate that closely mimic the real bone [1-2]. PLA with an extensive FDA approval history has been widely used in many applications such as screws, pins and plates for orthopedics [3-4], devices for controlled drug delivery [5-6] and also scaffolds for bone regeneration [7-8]. By incorporating HA to the PLA matrix has evidenced to enhance not only osteoinductivity and osteoconductivity but also mechanical properties of the composite material [9-10].

In this study, we focus on the fabrication of PLA-HA scaffolds and evaluation the ability of these scaffolds in supporting cell proliferation and differentiation. Since scaffold design is critically important in directing cells to form functional tissue, stereolithography (SLA)-a rapid prototyping technology- has been selected as a promising technique for the fabrication of complex shaped scaffolds. The photocurable liquid resin of PLA-HA composite was firstly prepared and then hardened by light, layer-by-layer, to form three dimensional scaffolds with precisely controlled porosity and pore size distribution. Then, the proliferation, alkaline phosphatase activity and mineralization of pre-osteoblasts on different scaffolds were examined to assess the potential application of these scaffolds in bone repair application.

2. Materials and Methods

2.1. Synthesis and scaffold fabrication

The DL-lactide oligomer was synthesized using the freshly prepared ethylene glycoxide as an initiator. The reaction was kept under vacuum at 70 °C for 2 days, and then raised to 100 °C for 3 days. After that, the oligomer was end-capped by reacting with excessive amount of methacrylic anhydride (MA) for 2 h at 120 °C under nitrogen. The number average molecular weight (Mn) and degree of methacrylation of oligomer were characterized by proton nuclear magnetic resonance (^{1}H NMR, Bruker DPX-300 spectrometer). The resulting methacrylated oligomer (PLA) was then prepared as photoactive composite resins (PLA-HA) by blending with triethylene glycol dimethacrylate (TEGDMA), UV initiator and HA.

A prototype SLA machine equipped with a 1.5 W UV laser source (355 nm wavelength) with 70 μm in diameter of laser spot size, constructed by MTEC, Thailand was used to fabricate 3D scaffolds with varying pore size from the prepared PLA-HA resins. The fabricated scaffolds were washed with isopropyl alcohol for several minutes, post-cured in a UV radiation oven for 1 h, and boiled in water at 100 ºC for 1 h before sterilization by gamma radiation (25 kGy). Stereomicroscope (ZEISS Model Stemi 2000) was used to view the structure of fabricated PLA-HA scaffolds with varying pore size.

2.2. Cell culture

The MC3T3-E1 pre-osteoblasts (ATCC CRL-2593) were used in the study. The cells were cultured in α-MEM supplement with 10%FBS, 1% penicillin/streptomycin, 10 mM *β*-glycerol phosphate and 100 μM L-ascorbic acid at 37^{O}C with 5% CO_2. After 80% confluence, the cells were seeded onto the sterilized PLA-HA scaffolds at concentration of 1×10^6 cells/specimen and then incubated for 3 h to allow the cells to attach. Afterwards, the culture medium was added onto the specimen and further cultured for 21 days. The culture medium was regularly refreshed every 2 days.

2.3. Cell proliferation

After a 21-day incubation period, cell proliferation on each scaffold was assessed by an alamar blue assay,

which is based on the detection of metabolic activity of the cultured cells. Briefly, the cells were incubated in a medium containing resazurin dye for 4 h. Aliquots of 200 µl of each medium were subsequently read for fluorescence intensity at the wavelength of 530/590 nm. Analysis of each sample was performed in triplicate.

2.4. Morphological analysis

The morphology of cells cultured on each scaffold was observed by scanning electron microscope (SEM) (Hitachi S-3400N). After incubation, the cell-seeded scaffolds were fixed with 2.5% (v/v) glutaraldehyde solution for 1 h at 4 ^{O}C, washed repeatedly with phosphate buffer saline (PBS), and then dehydrated at room temperature in an ethanol gradient series up to 100%. The scaffolds were subsequently subjected to critical point-dried with CO_2. The specimens were sputter-coated with gold and observed at 500x magnification with SEM.

2.5. Alkaline phosphatase (ALP) activity

ALP activity was determined by using alkaline phosphatase assay kit (ab83369, Abcam). This assay was performed to assess the ALP production of MC3T3-E1 pre-osteoblasts in the scaffolds. Briefly, the cell-seeded scaffolds were lyzed to obtain cell lysate solution. A 80 µl of solution was reacted with 50 µl of the 5 mM *p*-nitrophenyl phosphate as the substrate for 60 minutes before ending the reaction with the stop solution. After that the absorbance of sample was measured at 405 nm on a plate reader. All samples were calculated for ALP activity (U/ml) against a calibration curve of *p*-nitrophenol standards. Analysis of each sample was performed in triplicate.

2.6. Mineralization assay

The cell mineralization was performed by Alizarin Red assay using Osteogenesis Assay Kit (ECM815, Millipore). After 21 days, the cell-seeded scaffolds were fixed with 2.5% (v/v) glutaraldehyde for 15 minutes at room temperature. The specimens were washed with excess deionized water and then incubated with 400 µl of 40 mM Alizarin Red Stain Solution. After 20 min of incubation, the specimens were repeatedly washed with deionized water to remove the unincorporated dye. Afterwards, the stained specimens were incubated in the 10% acetic acid (v/v) solution for 30 min and further heated at 85 ^{O}C for 10 min. The resulting samples were subjected to centrifugation at 20,000×g for 15 min. A 120 µl of each sample solution was measured at the absorbance of 405 nm. The scaffold without cells was used as a background control. The samples were calculated for the amount of alizarin red (µM) against the calibration curve of Alizarin Red standards. Analysis of each sample was performed in triplicate.

3. Results and Discussion

3.1. 3.1. Synthesis and characterization

Oligolactide was synthesized via ring opening polymerization of D,L-lactide using ethylene glycoxide as an initiator and then end-functionalized with an excessive amount of MA to provide reactive sites for photo-crosslinking as illustrated in Figure 1. The number average molecular weight (Mn) and degree of methacrylation of the synthesized PLA determined by NMR were 1240 g/mol and 48%, respectively.

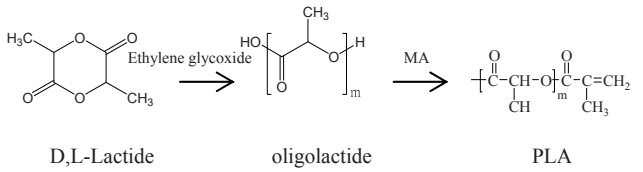

Figure 1. Synthesis of PLA.

PLA were then blended with photoinitiator, and TEGDMA as a reactive diluent at 50% w/w. HA at 55 and 75% were also blended into the resin matrix to obtain PLA-HA composites with improved mechanical and biological properties. Scaffolds with pore 1000 μm (M1000) and 1500 μm (M1500) fabricated by SLA are shown in Figure 2. The body-centered cubic scaffolds that designed to entrap seeded cell were formed nicely with interconnected structure indicating the powerful technique of SLA for fabricating the complex scaffolds. Three types of scaffolds were constructed, which were M1000 with 55 and 75% HA, and M1500 with 55% HA to observe their effects on cellular behavior.

3.2. Biological study

Figure 3 shows the fluorescence intensity of MC3T3-E1 pre-osteoblasts cultured on the three types of PLA-HA scaffolds after 21-day culture period. As seen from the intensity, it implied that all tested scaffolds, i.e. M1500-55 HA, M1000-55 HA, and M1000-75 HA, were non-cytotoxic to cells and could support the cell proliferation. Furthermore, the intensity results also showed no significant difference in cell number among them.

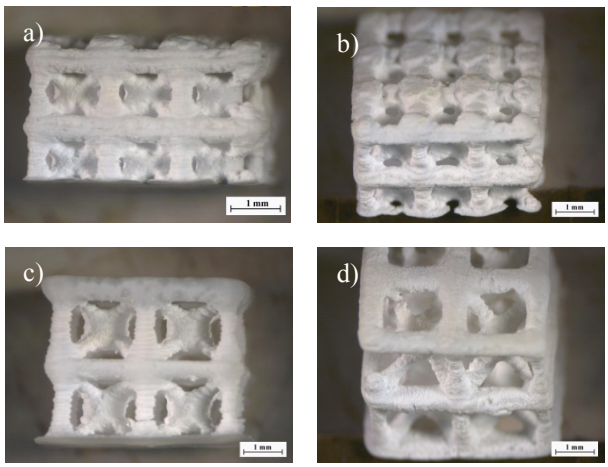

Figure 2. Scaffolds fabricated by SLA with pore size of 1000 μm (a,b) and 1500 μm (c,d); side view (a,c) and top view (b,d).

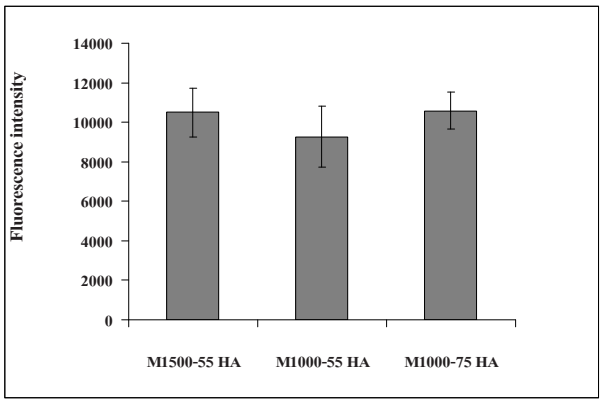

Figure 3. Cell proliferation on various PLA-HA scaffolds at a 21-day culture period.

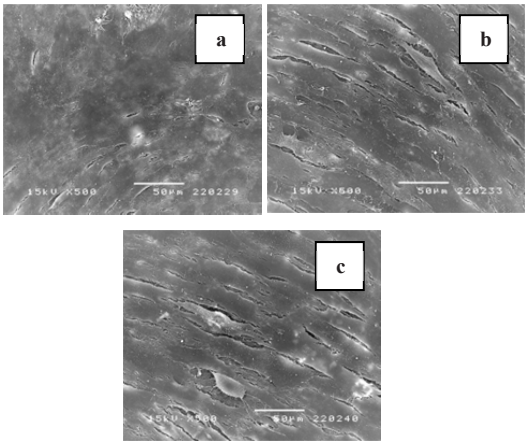

Figure 4. SEM images of the cell morphology on various PLA-HA scaffolds at a 21-day culture period; (a) M1500-55 HA, (b) M1000-55 HA, (c) M1000-75 HA, original magnification ×500.

The morphology and distribution of seeded cells on the scaffolds were evaluated by SEM, as shown in Figure 4. After 21 days of incubation, a large number of cells on all scaffold surfaces were vividly observed. Additionally, most cells appeared well spread and distributed uniformly throughout the scaffolds. This was in a good accordance with cell proliferation result.

Alkaline phosphatase (ALP) activity, an early marker of osteoblast phenotype, is up-regulated at the early state of osteoblastic differentiation and down-regulated during the time course of differentiation. In this study, the ALP activity of MC3T3-E1 pre-osteoblasts on each scaffold was assayed at 7 days of cultivation in order to determine cell differentiation. As revealed in Figure 5, the M1000-75 HA scaffold showed a considerably greater ALP activity than M1000-75 and M1500-55, respectively. This was attributed to the effect of different pore size and HA content in each type of scaffold on the ALP activity. Higher HA content in the scaffold could promote the ALP activity. In addition, the scaffold with smaller pore size provided a favorable environment for the activity.

The mineralization, a marker of cell differentiation, of cells cultured on the scaffolds was evaluated by alizarin red assay. After 21-day culture period, the calcium deposition by cells showed no significant difference among three types of PLA-HA scaffolds, as shown in Figure 6. It indicated that, at the early culture, the different characteristics of scaffold, i.e. the amount of incorporated HA and its pore size had an effect on the differentiation process, in terms of ALP activity, of MC3T3-E1 pre-osteoblasts towards osteoblastic lineage. However, after longer period of incubation (21 days) all types of scaffolds could equally support the proliferation of cells as well as their differentiation.

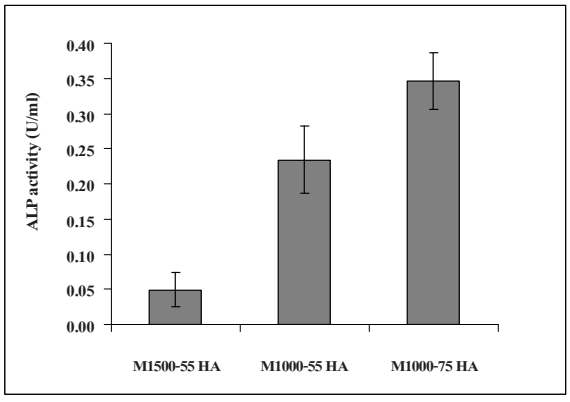

Figure 5. Intracellular ALP activity of cells on various PLA-HA scaffolds at a 7-day culture period.

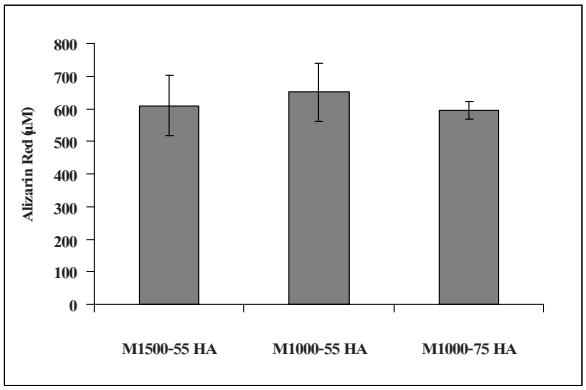

Figure 6. Alizarin red assay of cells culture on various PLA-HA scaffolds at a 21-day culture period.

4. Conclusions

The complex shaped scaffolds were successfully prepared from the PLA-HA resin by the technique of SLA. The HA amount and pore size affected the scaffold bioactivity, in terms of ALP, at the early state of osteoblastic differentiation. For the longer culture period, all scaffolds appeared to provide appropriate conditions to support the growth of cells and their differentiation, making them potentially suitable for bone tissue engineering.

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References

[1] Daculsi, G., Goyenvalle, E., Cognet, R., Aguado, E., Suokas, E.O., 2011. Ostoconductive properties of poly(96L/4D-lactide)/beta-tricalcium phosphate in long term animal model, Biomaterials 32, p.3166.

[2] Roether, J.A., Boccaccini, A.R., Hench, L.L., Maquet, V., Gautier, S., Jerome, R., 2002. Deveopment and invitro characterization of novel bioresorbable and bioactive composite materials based on polylactide foams and Bioglass for tissue engineering applications, Biomaterials 23, p.3871.

[3] Raghoebar, G.M., Liem, R.S.B., Bos, R.R.M., van der Wal, J.E., Vissink, A., 2006. Resorbable screws for fixation of autologous bone grafts, Clinical Oral Implants Research 17, p.288.

[4] Acosta, H.L., Stelnicki, E.J., Rodriguez, L., Slingbaum, L.A., 2005. Use of absorbable poly(D,L) lactic acid plates in cranial-vault remodeling: presentation of the first case and lessons learned about its use, The Cleft Palate-Craniofacial Journal 42, p.333.

[5] Ouchi, T., Saito, T., Kontani, T., Ohya, Y., 2004. Encapsulation and/or release behavior of bovine serum albumin within and from polylactide-grafted dextran microspheres, Macromolecular Bioscience 4, p.458.

[6] Singh, M., Shirley, B., Bajwa, K., Samara, E., Hora, M., Ohagan, D., 2001. Controlled releare of recombinant insulin-like growth factor from a novel formulation of polylactide-co-glycolide microparticles, Journal of Controlled Release 70, p.21.

[7] Melchels, F.P.W., Feijen, J., Grijpma, D.W., 2009. A poly (D,L-lactide) resin for the preparation of tissue engineering scaffolds by stereolithography, Biomaterials 30, p. 3801.

[8] Zhang, R., Ma, P.X., 2004. Biomimetic polymer/apatite composite scaffolds for mineralized tissue engineering, Macromolecular Bioscience 4, p.100.

[9] Hong, Z., Zhang, P., He, C., Qiu, X., Liu, A., Chen, L., Chen, X., Jing, X., 2005. Nano-composite of poly(L-lactide) and surface grafted hydroxyapatite: Mechanical properties and biocompatibility, Biomaterials 26, p.6296.

[10] Shim, J.H., Moon, T.S., Yun, M.J., Jeon, Y.C., Jeong, C.M., Cho, D.W., Huh, J.B., 2012. Stimulation of healing within a rabbit calvarial defect by a PCL/PLGA scaffold blended with TCP using solid freeform fabrication technology, Journal of Materials Science: Materials in Medicine 23, p. 2993.